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Vascular Endothelial Cells Produce Soluble Factors That Mediate the Recovery of Human Hematopoietic Stem Cells after Radiation Injury

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ABSTRACT

The risk of terrorism with nuclear or radiologic weapons is considered to be high over the coming decade. Ionizing radiation can cause a spectrum of hematologic toxicities, from mild myelosuppression to myeloablation and death. However, the potential regenerative capacity of human hematopoietic stem cells (HSCs) after radiation injury has not been well characterized. In this study, we sought to characterize the effects of ionizing radiation on human HSCs and to determine whether signals from vascular endothelial cells could promote the repair of irradiated HSCs. Exposure of human bone marrow $CD34^+$ cells to 400 cGy caused a precipitous decline in hematopoietic progenitor cell content and primitive cells capable of repopulating nonobese diabetic/severe combined immunodeficient mice (SCID-repopulating cells), which was not retrievable via treatment with cytokines. Conversely, culture of 400 cGy–irradiated bone marrow $CD34^+$ cells with endothelial cells under noncontact conditions supported the differential recovery of both viable progenitor cells and primitive SCID-repopulating cells. These data illustrate that vascular endothelial cells produce soluble factors that promote the repair and functional recovery of HSCs after radiation injury and suggest that novel factors with radiotherapeutic potential can be identified within this milieu.

KEY WORDS

Hematopoietic stem cells • Vascular endothelial cells • Radiation injury • Rescue

INTRODUCTION

Increasing levels of exposure to ionizing radiation can cause a spectrum of damage to the skin and the hematopoietic, gastrointestinal, pulmonary, and central nervous systems [1-4]. The hematopoietic and immune systems are among the most sensitive tissues to the adverse effects of ionizing radiation: lymphocyte decline and thrombocytopenia are reported after as low as 50 cGy of exposure [4]. After 400 cGy of exposure, more severe myelosuppression occurs, and the mortality risk is estimated to be 50% in the absence of medical intervention [4]. At doses >400 cGy, bone marrow (BM) failure and death can occur despite maximal supportive care with transfusion support and antibiotics [2-4].

Experimental studies have demonstrated that low-dose ionizing radiation induces cellular apoptosis via activation of Fas ligand–mediated pathways [5,6], whereas higher-dose radiation induces double-stranded DNA damage, which causes necrotic cell death in proliferating cells [7]. It is interesting to note that the administration of interleukin (IL)–1 or stem cell factor (SCF) before or at the time of high-dose radiation exposure protects mice from radiation lethality, thus suggesting that induction of stem/progenitor cells into late S phase of the cell cycle is radioprotective [8,9]. Alternately, administration of tumor necrosis factor α, which induces production of free-radical scavengers, is also radioprotective [10], although its efficacy is evident primarily after low-dose radiation exposure [11]. Administration of megakaryocyte growth and development factor (MGDF), a ligand for Mpl [12], at the time of high-dose irradiation is also 100% radioprotective in mice and has been shown to inhibit the actions of p53 to prevent radiation-induced apoptosis [12]. The combined administration of SCF, fms-like tyrosine kinase

3 (Flt-3) ligand, thrombopoietin (TPO), IL-3, and stromal cell derived factor 1 (SDF-1) to B6D2F1 mice within 2 hours after 800 cGy has also been shown to support the survival of 87.5% of mice, compared with 8.3% in controls [13]. However, in the event of a nuclear blast or a nuclear power plant accident, the administration of cytokines to victims within 2 hours of exposure will be difficult. Moreover, experimental studies have yielded conflicting results with regard to the potential benefits of cytokine administration when they are administered more than 2 to 4 hours after high-dose radiation exposure [9,14-18]. For example, Macvittie et al. [14] demonstrated that the administration of 10 μg/kg/d of granulocyte colony-stimulating factor plus supportive care beginning at 20 hours after 500 cGy of total body irradiation was associated with 75% survival of dogs, compared with 0% survival in untreated animals. Conversely, Zsebo et al. [9] demonstrated that administration of 100 μg/kg SCF beginning 4 hours after a lethal dose (1150 cGy) of total body irradiation in mice provided no radioprotection in any animals, and Neelis et al. [16] showed that the radioprotective effects of thrombopoietin were dramatically reduced between 2 and 24 hours after 600 cGy of exposure in mice. Taken together, these data indicate that additional therapies capable of accelerating hematopoietic reconstitution several hours to days after radiation-induced aplasia should be explored.

We examined the capacity of primary vascular endothelial cells (ECs) to support the self-renewal and expansion of murine, primate, and human hematopoietic stem cells (HSCs) [19-21]. In addition to the contribution of osteoblasts in supporting HSCs within the BM niche [22,23], the potential role of ECs in the BM vascular niche has recently been suggested [24]. We have observed that primary human brain ECs (HUBECs) support, in noncontact culture, a 1 to 2 log expansion of human BM and cord blood (CB) severe combined immunodeficient (SCID)-repopulating cells (SRCs) [25,26]. We also have observed that HSCs harvested from the BM of lethally irradiated C57BL/6 mice could be functionally rescued via coculture with brain ECs [19]. In this study, we examined whether human HSCs could be rescued from the deleterious effects of ionizing radiation via coculture with primary HUBECs. We found that soluble factors elaborated by HUBECs support the recovery and expansion of irradiated human BM HSCs, whereas treatment with cytokines alone is ineffective.

METHODS

HUBEC Cultures

HUBECs (passage >10) were developed in primary culture from explanted cortical brain vessel segments (obtained via autopsy specimens from the University of California-Los Angeles Department of Neuropathology) as previously described [21]. These cells highly express human von Willebrand factor, thus indicating an endothelial phenotype (data not shown). Briefly, gelatin-coated 6-well plates (Costar, Cambridge, MA) were seeded with 1×10^5 HUBECs in complete EC medium containing Medium 199 (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 0.3 mg/mL L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (1% penicillin/streptomycin), 60 mg/L EC growth supplement, and 4.5 U/mL heparin (Sigma, St. Louis, MO). HUBECs were cultured for 48 hours to >90% confluence in a 37°C, 5% carbon dioxide atmosphere before the establishment of $CD34^+$ cell cocultures.

Irradiation of Human $CD34^+$ Cells and In Vitro Coculture

Cryopreserved human BM $CD34^+$ (Cambrex, Gaithersburg, MD) or CB $CD34^+$ cells (AllCells, Berkeley, CA) were thawed, washed once, and resuspended at 1×10^6/mL in Iscove modified Dulbecco medium (IMDM; Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. $CD34^+$ cells (>95% purity) were then exposed to 400 cGy in vitro in polystyrene conical tubes (Becton Dickinson, San Jose, CA) by using a cesium 137 radiation source. Cells were maintained on ice and placed into culture 2 hours after irradiation. A dose of 400 cGy was used because this is a representative level of exposure that has been estimated to occur after nuclear power plant accidents [27].

Cultures were established with 1 to 2×10^5 irradiated BM or CB $CD34^+$cells in 6-well plates with media containing IMDM, 10% FBS, 1% penicillin/streptomycin, 20 ng/mL thrombopoietin, 120 ng/mL SCF, and 50 ng/mL fms-like Flt-3 ligand (TSF; R&D Systems, Minneapolis, MN). For noncontact HUBEC cocultures, irradiated BM or CB $CD34^+$ cells were placed into 0.4-μm polystyrene transwell inserts (Costar). Cultures were maintained in a 37°C, 5% carbon dioxide atmosphere for 10 days, with media supplementation (2 mL per well) at day 7. At day 10, nonadherent cells were collected from the culture by vigorous flushing with warm IMDM containing 10% FBS and 1% penicillin/streptomycin.

In Vitro Hematopoietic Progenitor Cell Assays

BM $CD34^+$ and CB $CD34^+$ cells that were irradiated in vitro with 400 cGy were analyzed for immunophenotype at 6 hours after irradiation. Day 0 nonirradiated cells were analyzed as controls. Irradiated cell subsets were also placed in culture with TSF or HUBECs under contact and noncontact conditions

approximately 4 hours after irradiation. Day 10 cultured progeny were collected and washed with phosphate-buffered saline (Invitrogen) and resuspended in IMDM with 10% FBS and 1% penicillin/streptomycin. The total viable cell count was determined by hemacytometer count with trypan blue dye exclusion. For phenotype analysis, cells were stained with anti-CD34 fluorescein isothiocyanate and anti-CD38 phycoerythrin or the appropriate immunoglobulin G isotype control antibodies (Becton Dickinson) for 30 minutes on ice. For apoptosis analysis, cells were stained with anti-annexin (Becton Dickinson) V fluorescein isothiocyanate, anti-CD38 phycoerythrin, and anti-CD34 allophycocyanin for 30 minutes on ice. Cells were washed twice and stained with 7-aminoactinomycin D (7-AAD; Becton Dickinson) for 10 minutes on ice before analysis. Sample acquisition was conducted on a FACScalibur flow cytometer (Becton Dickinson). Statistical comparisons between groups were performed by using the *t* test.

Colony-forming assays were established in MethoCult GF H4434 complete methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) with 1×10^3 cells per dish in 35-mm gridded petri dishes (Nunc, Rochester, NY), according to the manufacturer's recommended protocol. After 14 days, triplicate cultures were scored for burst-forming units-erythroid (BFU-E), colony-forming units-granulocyte monocyte (CFU-GM), and colony-forming unit-mix (CFU-Mix) colony (>50 cells) formation.

Nonobese Diabetic/SCID Repopulation Assays

Six- to 8-week-old nonobese diabetic/SCID (NOD/SCID) mice [28] underwent transplantation with day 0 400 cGy–irradiated BM $CD34^+$ cells ($0.75\text{-}1.5 \times 10^6$) or their cultured progeny. A subset of mice was also injected with an identical dose of normal, day 0 nonirradiated BM $CD34^+$ cells as a positive control. NOD/SCID mice received transplants via tail vein injection after receiving 300 cGy of total body irradiation on an X-Rad 320 irradiation system (AGFA NDT Inc., Lewistown, PA) at a dose rate of 100 cGy/min 4 hours before transplantation, as previously described [26]. Eight weeks after transplantation, mice were killed, and marrow was collected from bilateral femurs by flushing with cold Dulbecco's phosphate buffered saline with 10% FBS. Red cells were lysed by using red blood cell lysing buffer (Sigma) and washed twice, and flow cytometric analysis was performed to determine human hematopoietic engraftment by using monoclonal antibodies against human leukocyte differentiation antigens to identify engrafted human leukocytes and discriminate their hematopoietic lineages [21,29]. Mice were scored as positively engrafted if the BM displayed ≥0.1% human $CD45^+$ cells via high-resolution flow cytometry analysis, consistent with previously published criteria for human cell repopulation in NOD/SCID mice [30,31].

RESULTS

Contact and Noncontact Culture with HUBECs Increases the Recovery of Irradiated Hematopoietic Progenitors

The combination of thrombopoietin, SCF, and Flt-3 ligand (TSF) has been shown to optimize the in vitro maintenance of CB SRCs [32,33], and our group has shown that these same cytokines, when combined with HUBECs, maximize the ex vivo expansion of purified BM $CD34^+CD38^-$ SRCs [26]. Therefore, we chose to compare the capacity for TSF alone versus HUBEC plus TSF to support the recovery of BM and CB $CD34^+$ cells after irradiation with 400 cGy.

Culture of 400 cGy–irradiated BM $CD34^+$ cells with TSF alone supported a 2.8-fold increase in total viable cells compared with day 0 cells; however, a significant decrement in the $CD34^+CD38^-$ subset was observed by day 10 (Figure 1A and B). Conversely, HUBEC contact cultures supported an 11.3-fold increase in total cells at day 10 and were associated with a significant increase in the percentage of $CD34^+CD38^-$ cells in culture (mean, 18.2%) compared with TSF alone (mean, 0.5%; $P = .005$). This translated into a 29.4-fold increase in $CD34^+CD38^-$ cells in HUBEC contact cultures compared with input, as compared with a 4.9-fold decrease in $CD34^+CD38^-$ cells with cytokines alone. Culture of irradiated BM $CD34^+$ cells with HUBECs under noncontact conditions supported a 5.8-fold expansion of total cells and a 4.8-fold increase in the $CD34^+$ $CD38^-$ subset compared with day 0 cells (Figure 1A and B). Although this was significantly less than the recovery observed in HUBEC contact cultures, the recovery of total viable cells and $CD34^+CD38^-$ cells in noncontact HUBEC cultures was significantly increased compared with TSF cultures alone ($P = .01$ and $P = .01$, respectively).

Irradiation of human CB $CD34^+$ cells yielded similar results as compared with BM $CD34^+$ cells. After a 10-day culture with TSF alone, a 9.2-fold expansion of total cells was observed, but a 3.9-fold decline in $CD34^+CD38^-$ cells occurred by day 10 (Figure 1C and D). In contrast, HUBEC contact cultures supported a 29.5-fold increase in total cells and a 28.6-fold increase in $CD34^+CD38^-$ cells compared with input. Noncontact HUBEC cultures supported a 17.7-fold increase in total cells and a 3.9-fold increase in $CD34^+CD38^-$ cells (Figure 1C and D). The recovery and expansion of total viable cells and $CD34^+CD38^-$ cells was significantly higher in both HUBEC contact and noncontact cultures compared

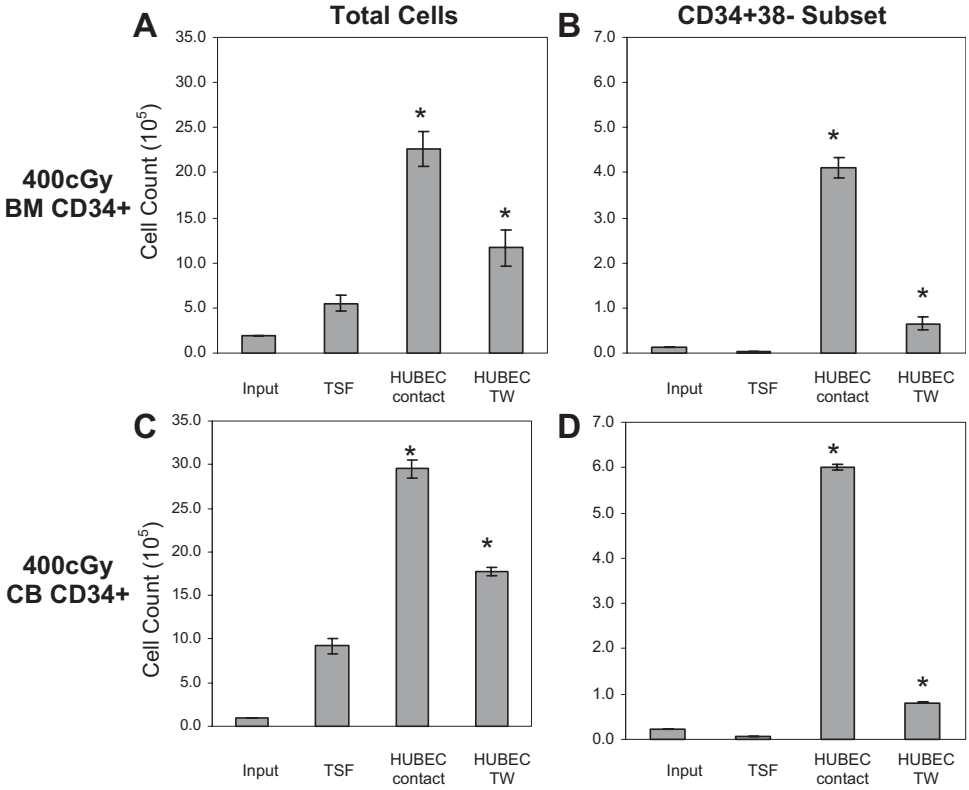

Figure 1. HUBEC culture supports the recovery of irradiated human hematopoietic progenitor cells. Primary human BM $CD34^+$ cells were irradiated in vitro with 400 cGy and placed in culture with TSF alone, HUBEC contact culture, or HUBEC noncontact (transwell; TW) culture. The mean recovery of total cells (A) and $CD34^+CD38^-$ cells (B) is shown at the top and demonstrates significantly improved recovery of both populations via coculture with HUBECs under contact and noncontact conditions as compared with TSF alone. Similarly, the recovery of total cells (C) and $CD34^+CD38^-$ cells (D) within 400 cGy–irradiated CB $CD34^+$ cells was also significantly greater after both contact and noncontact HUBEC culture as compared with TSF alone. *The mean number of cells in the identified condition is significantly different from that in the TSF culture group.

with cultures with TSF alone ($P \leq .01$). Figure 2 shows a representative phenotypic analysis of day 0 BM and CB $CD34^+$ cells after 400 cGy of irradiation and their progeny after culture with TSF alone and HUBEC contact and noncontact cultures.

Coculture with HUBECs Supports the Recovery of Colony-Forming Cells from Irradiated BM and CB

Colony-forming cell (CFC) assay of normal and 400 cGy–irradiated day 0 BM and CB $CD34^+$ cells highlighted the ablative effects of 400 cGy of ionizing radiation on hematopoietic progenitor cell activity (Figure 3). The 400 cGy–irradiated BM $CD34^+$ cells contained 18.4-fold less CFC content (CFU-total; $P < .001$) and showed marked reductions in BFU-E (6.9-fold reduction) and CFU-GM (32.7-fold reduction) content and a complete loss of CFU-Mix colonies, as compared with nonirradiated BM $CD34^+$ cells. Significant reductions in CFU-total ($P = .008$; Figure 3), BFU-E, CFU-GM, and CFU-Mix content were also observed after irradiation of CB $CD34^+$ cells.

Both contact and noncontact HUBEC cultures supported the recovery of CFCs from 400 cGy–irradiated BM $CD34^+$ cells at levels significantly greater than TSF alone (3.6-fold and 3.9-fold increased CFU-total, respectively; $P < .001$ and $P = .002$). HUBEC contact and noncontact cultures also recovered BFU-E and CFU-Mix colonies, which were completely absent from TSF-cultured progeny of irradiated BM $CD34^+$ cells (data not shown). HUBEC contact and noncontact cultures of 400 cGy–irradiated CB $CD34^+$ cells yielded similar results, with significant increases in CFU-total content (3.2-fold and 3.0-fold; $P = .02$ and $P < .005$, respectively), as well as BFU-E, CFU-GM, and CFU-Mix (data not shown), as compared with TSF alone (Figure 3).

Coculture with HUBECs Reduces Hematopoietic Progenitor Cell Death after Radiation Exposure

We hypothesized that ECs might elaborate antiapoptotic factors that could promote the recovery of hematopoietic progenitor cells after radiation injury. Analysis with Annexin V and 7-AAD revealed interesting similarities and differences in the percentage of apoptotic (Annexin V^+/7-AAD^-) and necrotic (Annexin V^+/7-AAD^+) cells within the nonirradiated BM

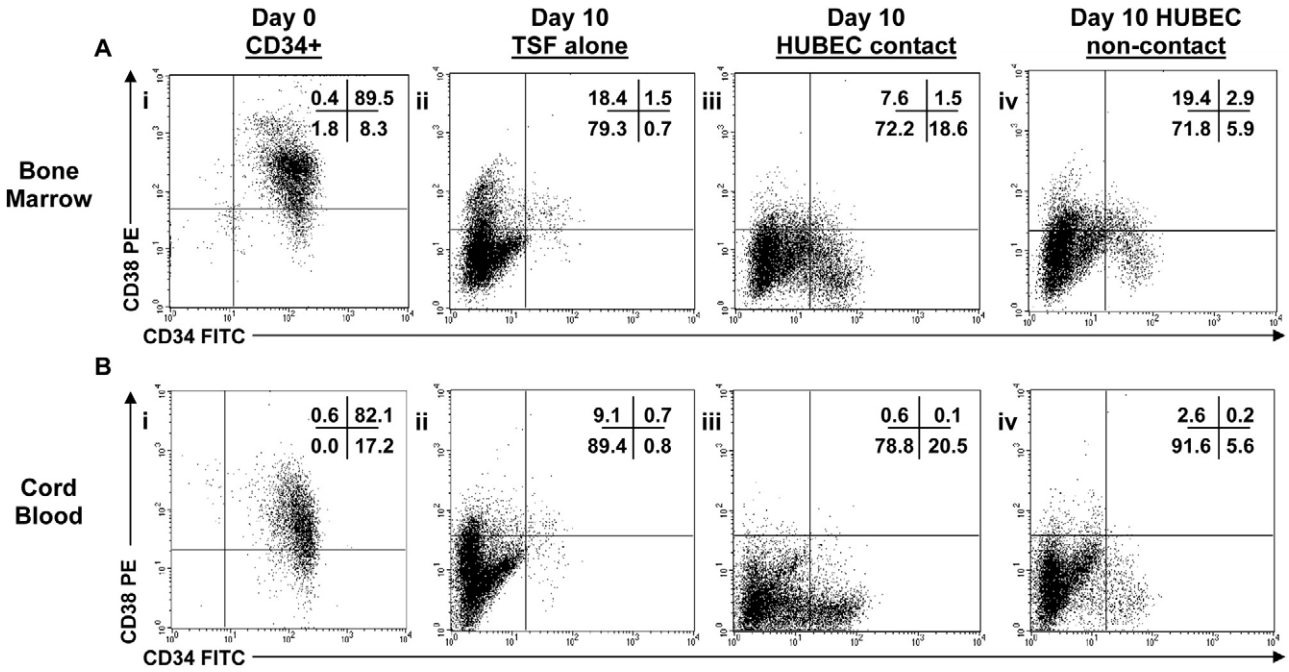

Figure 2. HUBEC coculture maintains a higher percentage of $CD34^+CD38^-$ cells after radiation injury than TSF alone. The 400 cGy–irradiated BM and CB $CD34^+$ cells were placed in 10-day cultures and analyzed by flow cytometry to determine phenotype changes. A representative analysis of day 0 400 cGy–irradiated BM $CD34^+$ cells is shown (Ai), along with analysis of the day 10 progeny of TSF culture, revealing a nearly complete loss of $CD34^+CD38^-$ cells (Aii). In contrast, HUBEC contact (Aiii) and HUBEC noncontact (Aiv) cultures maintained a population of $CD34^+CD38^-$ cells at day 10. Similar maintenance of CB $CD34^+CD38^-$ cells after 400 cGy was also observed during HUBEC contact and noncontact cultures (B). The percentages of cells in each quadrant are shown in the upper right of each figure. FITC indicates fluorescein isothiocyanate.

$CD34^+$ cells, the irradiated BM $CD34^+$ cells, and the progeny of irradiated BM $CD34^+$ cells under different culture conditions. Notably, overall cell death was significantly increased within 400 cGy–irradiated BM $CD34^+$ cells measured 6 hours after irradiation as compared with nonirradiated BM $CD34^+$ cells (Figure 4). Analysis of the entire population of irradiated BM cells demonstrated moderately increased apoptosis and necrosis in TSF cultures as compared with both HUBEC contact and noncontact cultures, and this was most evident at day 3 and day 10 (Figure 4A). Within the $CD34^+$ progenitor cell subset, TSF culture was associated with a significant increase in cell death over time as compared with both HUBEC contact and noncontact cultures (Figure 4B).

HUBEC Culture Supports the Recovery of Repopulating Stem Cells from Irradiated Human BM $CD34^+$ Cells

NOD/SCID mice received transplants via tail vein injection with day 0 normal (nonirradiated), day 0 400 cGy–irradiated, or the progeny of 400 cGy–irradiated BM $CD34^+$ cells after a 10-day culture with HUBECs or TSF alone. We observed that exposure to 400 cGy of ionizing radiation had a profoundly deleterious effect on the repopulating capacity of BM $CD34^+$ cells. Mice that underwent transplantation with a dose of 0.75×10^6 nonirradiated BM $CD34^+$ cells demonstrated low-level (mean, 0.1% human $CD45^+$ cells) engraftment in 50% of transplanted animals (Figure 5). Conversely, mice that underwent transplantation with day 0 400 cGy–irradiated BM $CD34^+$ cells or their day 10 progeny after culture with TSF alone demonstrated no human cell engraftment. Mice that underwent transplantation with the progeny of 0.75×10^6 400 cGy–irradiated BM $CD34^+$ cells after HUBEC contact culture also showed no human $CD45^+$ cell engraftment $\geq$0.1%, although a single mouse had 0.02% human $CD45^+$ cells at 8 weeks after transplantation.

At a dose of 1.5×10^6 nonirradiated BM $CD34^+$ cells, 100% of transplanted mice demonstrated human $CD45^+$ cell engraftment at high levels (mean, 36.8% human $CD45^+$ cells). Conversely, mice that underwent transplantation with 400 cGy–irradiated BM $CD34^+$ cells showed human $CD45^+$ cell engraftment in 75% of animals, with significantly lower levels of engraftment (mean, 1.0% human $CD45^+$ cells; Figure 5). This indicates that a small population of SRCs was able to survive 400 cGy of radiation injury. It is interesting to note that the progeny of the identical dose of 400 cGy–irradiated BM $CD34^+$ cells cultured with TSF alone for 10 days were incapable of engrafting and repopulating any transplanted mice; this suggests

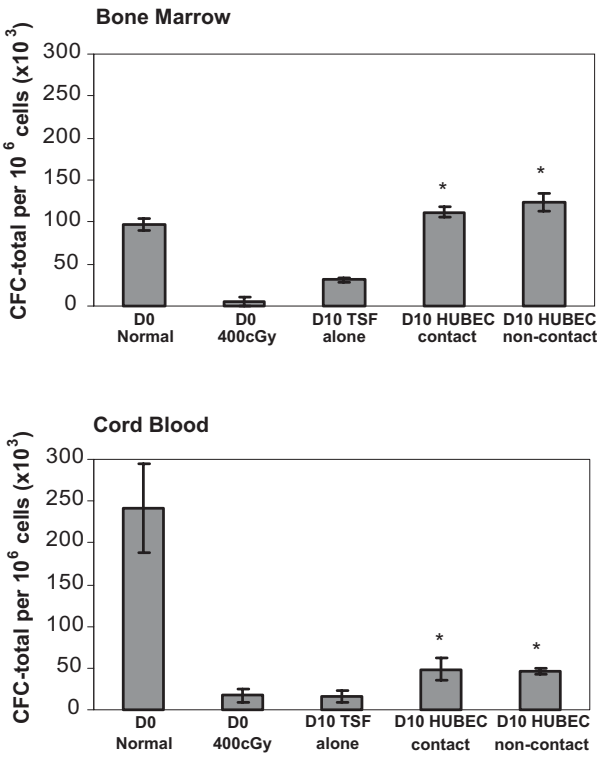

Figure 3. HUBEC contact and noncontact cultures promote the recovery of CFCs compared with TSF alone. Day 0 normal and 400 cGy–irradiated $CD34^+$ cells and the day 10 progeny of 400 cGy–irradiated $CD34^+$ cells after culture with TSF alone, HUBEC contact, and HUBEC noncontact culture were analyzed for CFC content after 14 days. The 400-cGy exposure caused a significant reduction in human CFC content at day 0. The progeny of 400 cGy–irradiated BM $CD34^+$ cells (A) and CB $CD34^+$ cells (B) after HUBEC contact and noncontact cultures contained significantly more CFU-total as compared with the progeny of 400 cGy–irradiated $CD34^+$ cells cultured with TSF alone. *The mean number of cells in the identified condition is significantly different from that in the TSF culture group. D indicates day.

that cytokine treatment was insufficient and possibly deleterious toward the survival of primitive long-term repopulating stem cells after high-dose radiation exposure. In contrast, the progeny of 1.5×10^6 400 cGy–irradiated BM $CD34^+$ cells cultured under noncontact conditions with HUBECs engrafted in 100% of transplanted mice with a mean engraftment level of 3.5% human $CD45^+$ cells per mouse, thus demonstrating that soluble endothelial factors promoted the recovery of irradiated human HSCs independently of cell-cell contact.

Representative phenotypic analyses of human $CD45^+$ cell frequencies in mice that underwent transplantation with nonirradiated BM $CD34^+$ cells, irradiated BM $CD34^+$ cells, and the progeny of 400 cGy–irradiated BM $CD34^+$ cells after culture with TSF alone versus noncontact HUBEC culture are shown in Figure 6A. Of note, mice that underwent transplantation with the progeny of 400 cGy–irradiated BM $CD34^+$ cells cultured with HUBECs under noncontact conditions demonstrated multilineage (B lymphoid and myeloid) engraftment, thus indicating that multipotent stem/progenitor cells were maintained after irradiation and HUBEC coculture (Figure 6B). Of note, the proportion of B lymphoid regeneration in mice that underwent transplantation with irradiated/HUBEC-cultured cells was comparatively higher than the observed regeneration of $CD13^+$ myeloid progeny, and this suggests a potentially important difference with regard to the native recovery of B lymphoid progenitors versus myeloid progenitors after high-dose irradiation.

DISCUSSION

Recently, because of the acknowledged risk of nuclear or radiological terrorism over the coming decade, there has been renewed interest in the development of medical countermeasures to the effects of ionizing radiation exposure [1-4]. Therapies directed at ameliorating the hematologic toxicity of ionizing radiation would be of particular interest because BM failure is the leading cause of death in victims of pure ionizing radiation injury [1-4,34,35]. In animal models, administration of cytokines such as SCF, MGDF, Flt-3 ligand, IL-1, or tumor necrosis factor α before or immediately at the time of high-dose total body radiation exposure can provide radioprotection and improve survival [8-12,16-18]. Similarly, the combined administration of multiple cytokines, including SCF, Flt-3 ligand, MGDF, IL-3, and SDF-1, within 2 hours after sublethal and near-lethal irradiation has been associated with decreased myelosuppression and improved survival in mice and baboons [13,36]. However, in the event of a radiologic or nuclear catastrophe, the administration of hematopoietic cytokines will not be feasible for many within the first 2 hours of exposure, and it is unclear whether the administration of cytokines more than a few hours after exposure would be therapeutically valuable after higher-dose exposures. SCF, for example, has no effect on radiation-induced myelosuppression and mortality when administered only 4 hours after high-dose radiation exposure [9], and granulocyte colony-stimulating factor, which clearly accelerates myeloid recovery in primates after sublethal exposures [37-40], has no effect when administered to mice after an exposure of 10.5 Gy [40]. Although we did not measure the capacity for HUBEC coculture to rescue human HSCs irradiated in vitro with doses >400 cGy, we have recently demonstrated that fully functional BM stem and progenitor cells can be rescued after harvest from lethally irradiated (1050 cGy of total body irradiation) C57BL/6 mice via coculture with porcine brain ECs [19]. Because few studies have been performed to

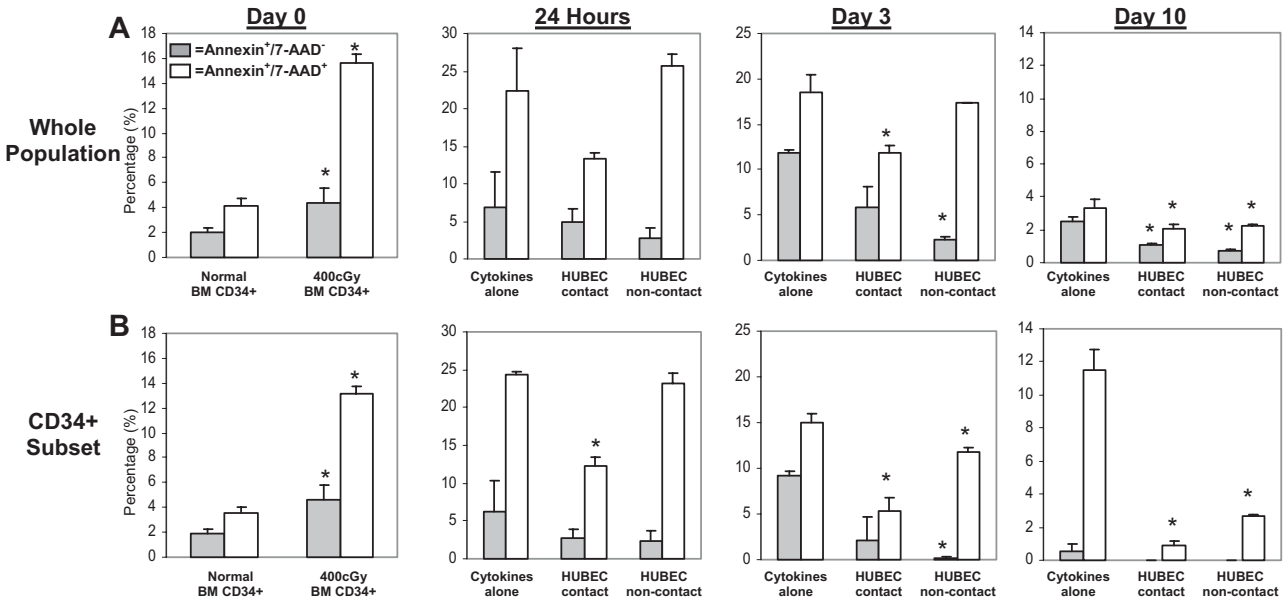

Figure 4. HUBEC coculture decreases hematopoietic progenitor cell death after radiation injury. Primary human BM $CD34^+$ cells (>95% purity) were irradiated with 400 cGy and subsequently placed in culture with TSF alone or HUBEC contact and noncontact cultures supplemented with TSF. Flow cytometric analysis was performed to measure the percentage of apoptotic and necrotic cells in each condition over time. Day 0 nonirradiated BM $CD34^+$ cells and 400 cGy–irradiated BM $CD34^+$ cells were analyzed as controls. A, Analysis of the entire population demonstrated that 400 cGy caused a significant increase in both apoptotic and necrotic cells by 6 hours after exposure ($P < .05$ for each comparison). By days 3 and 10, a modest but significant decrease in the percentage of apoptotic and necrotic cells was observed within HUBEC contact and noncontact cultures as compared with TSF alone ($P < .05$). B, Analysis of the $CD34^+$ progenitor cell subset over time demonstrated a more significant reduction in cell death within the HUBEC contact and noncontact cultures as compared with TSF alone ($P < .05$ for each comparison), thus suggesting a differential effect of endothelial cell culture on progenitor cell repair after radiation injury. *The mean percentage of cells in the identified condition is significantly different from that in the TSF culture group.

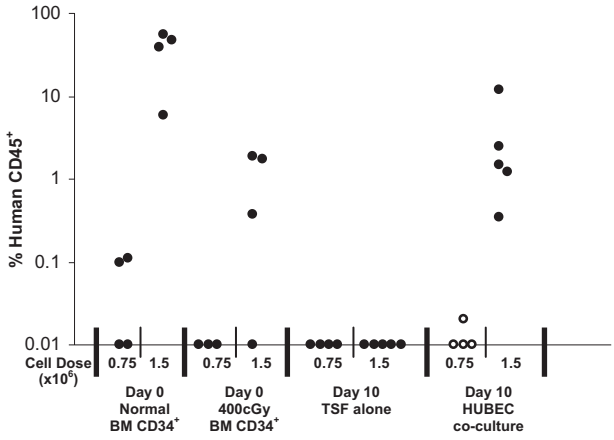

Figure 5. HUBEC coculture supports the recovery of human BM long-term repopulating cells after radiation injury. NOD/SCID mice underwent transplantation with 0.75 to 1.5 × 10^6 nonirradiated or 400 cGy–irradiated BM $CD34^+$ cells per mouse or the progeny of 400 cGy–irradiated BM $CD34^+$ cells after culture with TSF alone or HUBEC contact (open circles) and noncontact (filled circles) cultures. The dose of 400 cGy caused a marked reduction in day 0 SRC content, and culture of 400 cGy–irradiated BM $CD34^+$ cells with TSF alone was associated with a complete loss of SRC over time. Conversely, noncontact culture with HUBECs maintained SRC content, thus indicating that soluble factors produced by HUBECs contributed to HSC repair.

characterize the regenerative capacity of human HSCs after ionizing radiation exposure, we propose that the HUBEC coculture model has the potential to yield important insights regarding EC-derived factors that may be radioprotective.

In this study, we showed that primary human BM $CD34^+$ cells are exquisitely sensitive to 400 cGy of exposure in vitro, thus resulting in dramatic declines in CFC and SRC content after injury. Treatment with hematopoietic cytokines alone was associated with an increase in apoptosis and necrosis of irradiated BM $CD34^+$ cells and a marked decline in $CD34^+CD38^-$ cells and SRCs compared with input, despite treatment within 4 hours of exposure, thus suggesting that cytokine treatment of human BM HSCs after high-dose irradiation may, in fact, be deleterious to their recovery. These results are in contrast to the observations of Drouet et al. [27], who reported that early treatment of baboon BM $CD34^+$ cells with SCF, Flt-3 ligand, thrombopoietin, and IL-3 decreased Fas ligand–mediated apoptosis in vitro. However, in that study, the authors did not examine the long-term repopulating potential of the irradiated baboon cells after cytokine treatment, so conclusions regarding the effect of cytokines on baboon HSCs cannot be drawn [27]. In addition, ionizing radiation induces mammalian cells,

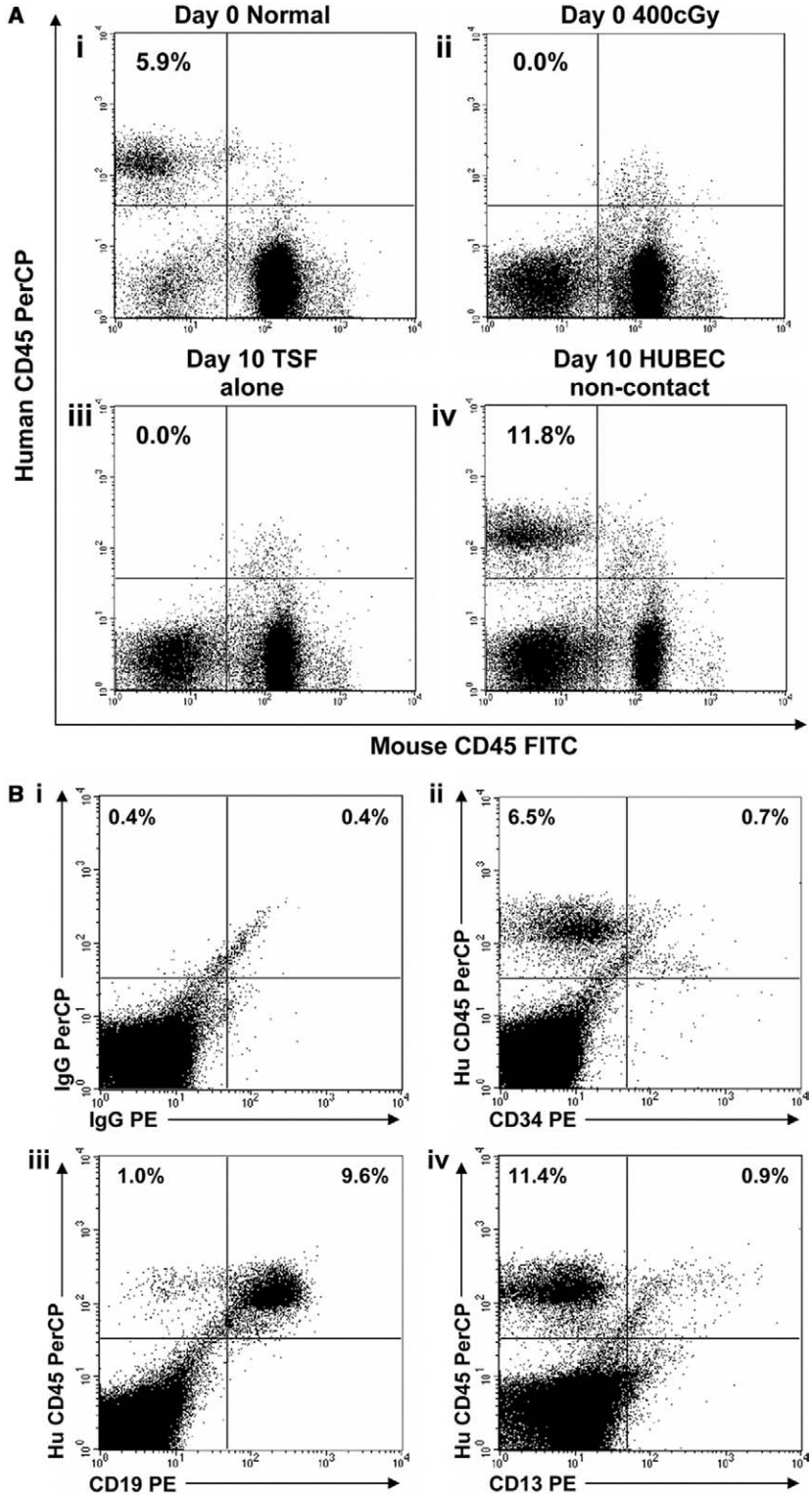

Figure 6. Noncontact culture of 400 cGy–irradiated BM $CD34^+$ cells with HUBECs maintains cells with multilineage differentiative capacity. A, Representative NOD/SCID BM analysis is shown from mice injected with 1.5×10^6 nonirradiated BM $CD34^+$ cells (Ai), 400 cGy–irradiated day 0 BM $CD34^+$ cells (Aii), or the progeny of the identical dose of 400 cGy–irradiated BM $CD34^+$ cells after a 10-day culture with TSF alone (Aiii) or HUBEC noncontact culture (Aiv). B, Multiparameter flow cytometric analysis was performed on engrafted human cells in representative mice. Isotype control staining is shown (Bi), and $CD34^+$ progenitor cells were demonstrated (Bii), as were $CD19^+$ B cells (Biii) and $CD13^+$ myeloid cells (Biv) in transplanted mice at 8 weeks. The relative proportion of B cells in the transplanted mice was increased as compared with myeloid cells; this indicates that lymphoid recovery may have occurred more rapidly after radiation injury. FITC indicates fluorescein isothiocyanate; PerCP, peridinin-chlorophyll protein complex.

in general, to undergo either cell-cycle arrest or apoptosis in the immediate postexposure period [41,42]. Cells that undergo p53-independent cell-cycle arrest in G_1 or G_2/M phase have the potential to repair radiation-induced DNA damage and avoid cell death [43]. Because the cytokine combination of thrombopoietin, SCF, and Flt-3 ligand has been shown to induce nearly 100% of human CB $CD34^+$ cells through at least 1 cell division by 1 week of culture [33], it is plausible that exposure of irradiated BM $CD34^+$ cells to these proliferation-inducing cytokines accelerates the demise of stem and progenitor cells. Conversely, HUBEC contact cultures and, to a lesser extent, HUBEC noncontact cultures decreased radiation-induced apoptosis and necrosis of BM $CD34^+$ cells and promoted a significant increase in the recovery of total viable cells, $CD34^+CD38^-$ cells, and CFCs as compared with TSF alone. These results are consistent with prior studies that suggested that that cell-cell contact interactions (eg, Jagged-Notch) between HSCs and other stromal cell types are critical to HSC survival [22,23,44].

It is interesting to note that although HUBEC contact cultures supported a greater recovery of total viable cells and $CD34^+CD38^-$ cells than noncontact cultures, HUBEC noncontact cultures supported a potent recovery of the most primitive SRCs. These data can be potentially explained, as others have shown [31], by the lack of correlation between stem cell content and phenotypic indicators after ex vivo culture. It is also possible that coculture with HUBECs induced cell-cycle arrest in HSCs as a mechanism of radioprotection, as has been described when murine embryonic neural stem cells were cultured in contact with murine brain ECs [45]. However, we have found that coculture with porcine brain ECs induces the proliferation of BM HSCs harvested from lethally irradiated mice [19], thus suggesting that a true expansion of radioprotected HSCs occurs via this strategy.

It is important to note that our results demonstrate that soluble factors produced by ECs support the survival and regeneration of human HSCs after radiation injury. Our previous analyses have shown that established hematopoietic cytokines, including SCF, Flt-3 ligand, thrombopoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and IL-3, are not enriched within HUBEC conditioned media [46]. This suggests that potentially novel prosurvival factors elaborated by HUBECs account for the effects we have observed. This soluble activity may be unique to vascular ECs as well. Recent studies by Mourcin et al. [47] and Drouet et al. [48] demonstrated that coculture with mesenchymal stem cells supported the recovery of 400 cGy–irradiated baboon $CD34^+$ cells, but this was dependent on cell-cell contact between the irradiated cells and the mesenchymal stem cells. We are currently pursuing identification of the HUBEC-soluble proteins that are responsible for the observed radiotherapeutic effects, via complementary genomic and protein fractionation strategies. We anticipate that the identification and characterization of these soluble proteins may facilitate the development of therapeutics to counteract radiation-induced myelosuppression.

The results presented here suggest that hematopoietic progenitor cells could, in principle, be collected from radiation-accident victims, expanded ex vivo, and transplanted in an autologous manner to accelerate the hematopoietic recovery of such victims. However, in the event of a mass casualty situation, such an approach would not be logistically feasible. Nonetheless, these data suggest an important contribution of vascular ECs to the repair and regeneration of human BM stem and progenitor cells after radiation injury. In other disease models, such as myocardial infarction and peripheral vascular disease, the antiapoptotic activity of circulating ECs has been proposed [49]. In addition, Kopp et al. [50] have shown the potential contribution of the BM vascular niche and angiogenic factors toward accelerating hematopoietic recovery after myelosuppressive chemotherapy. Targeted therapies aimed at accelerating the recovery of the BM vascular endothelial niche may therefore be of benefit in the treatment of victims of radiation injury. As proof of principle, we have recently observed that tail vein transplantation of primary vascular ECs is radioprotective and accelerates hematopoietic recovery in lethally irradiated mice (Chute J, unpublished data).

A recent workshop on radiation countermeasures sponsored by the National Institute of Allergy and Infectious Diseases concluded that the current lack of effective therapeutic agents for the treatment of radiation victims is a major problem in the government's preparation for radiologic or nuclear catastrophes [1]. More broadly, newly developed therapies that accelerate hematopoietic recovery after radiation injury could also have application in attenuating the myelotoxic effects of chemotherapy and radiotherapy in patients with cancer. Our results indicate that human vascular ECs elaborate soluble factors that support the repair and recovery of irradiated human stem and progenitor cells. The characterization of these novel factors has the potential to lead to targeted therapies for radiation-induced myelosuppression.

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